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Identification, Growth and Pathogenicity of *Colletotrichum boninense* Causing Leaf Anthracnose on Japanese Spindle Tree

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Leaf anthracnose was observed on leaves of Japanese spindle tree in Seoul, Korea from autumn 2003 to spring 2004. The causal fungus was purely isolated from the leaf spot lesions and cultured on PDA. The colony on PDA was cream to orange but blackish in the center on old cultures. Conidia were formed in blackish orange masses and were cylindrical in shape, measured 13-17 × 5-7 μm in size. Blackish brown setae were often observed on PDA and ranged up to 100 μm in length. Based on morphological and ITS region sequence analyses, the fungal strain was identified as *Colletotrichum boninense*. Koch's postulates were fulfilled by inoculating tree leaves with 1×10^6 conidia per ml in a moist chamber. This is the first study on the pathogenicity, growth and phylogenetic characteristics of *C. boninense* causing leaf anthracnose on Japanese spindle tree in Korea.

Keywords : *Colletotrichum boninense*, *Euonymus japonica*, ITS region sequence, leaf anthracnose

Colletotrichum (anamorph of *Glomerella*) consists of numerous phytopathogenic species that cause diseases in a wide range of hosts (Sutton, 1980). According to the taxonomic revision of *Colletotrichum* species by von Arx (1957), *C. gloeosporioides* had more than 600 synonyms and showed many morphological and physiological variations. Sutton (1992) described seven formae speciales of *C. gloeosporioides* and recognized the species as a heterogeneous group with a great variation in morphology, although some species were separated from *C. gloeosporioides* (Holliday, 1980; Sutton, 1980; van der Aa, 1978; von Arx, 1981). In addition, some new species with different morphology and/or pathogenicity have been reported and segregated from *C. gloeosporioides* (Shivas et al., 1998; Waller et al., 1993). *Colletotrichum boninense* previously fell within the broad species concept of *C. gloeosporioides*, but is differentiated from *C. gloeosporioides* by colony shape, conidial morphology and molecular phylogenetic analysis of ITS sequences (Moriwaki et al., 2003; Lu et al., 2004).

Recently, an isolate of a species of *Colletotrichum* was collected from Japanese spindle tree (*Euonymus japonica* Thunb.) leaves in Kwanak Mountain, Seoul, Korea. An abstracted form of its occurrence and characteristics was reported to the New Disease Reports to be published in Plant Pathology (Lee et al., 2005) and now the full text of the study is provided here. Some features of the isolate were different from those of *C. gloeosporioides*. Mahoney and Tattar (1980) reported *C. gloeosporioides* as an anthracnose pathogen on *Euonymus fortunei*. *Colletotrichum gloeosporioides* and *C. griseum* were once reported to occur on *E. japonica* or *Euonymus* sp. in the USA and Canada (Alfieri et al., 1984; Grand, 1985; Hilton, 2000; USDA, 1960). However, there have been no reports on the distribution and host ranges of *C. boninense* as well as *C. griseum* yet.

The purposes of this study were to compare our isolate with related species of *C. boninense* and *C. gloeosporioides* after previous descriptions, reveal its phylogenetic relationships with related species, and investigate the growth at different temperatures and pathogenicity to its host.

Materials and Methods

Fungal strains and cultures. From autumn 2003 to spring 2004, leaves of Japanese spindle tree with black leaf spot lesions (Fig. 1) were collected from Kwanak Mountain, Seoul, Korea. The causal fungus was isolated from the lesions using a standard blotter method (ISTA, 1976). The leaf surface was treated with 1% sodium hypochlorite for one minute and then the leaf tissues were put on PDA. Media used for cultivation of fungal strains were YMA (yeast malt agar; yeast extract 15 g, malt extract 15 g, agar 15 g l^{-1}), MEA (malt extract agar; malt extract 15 g, glucose 15 g, peptone 1 g, agar 15 g l^{-1}), PDA (potato dextrose agar; potato dextrose broth 24 g, agar 15 g l^{-1}), modified YpSs (yeast extract potassium phosphate soluble starch agar; yeast extract 2 g, soluble starch 7.5 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, agar 15 g l^{-1}), and WA (water agar; agar 20 g l^{-1}). The fungal strains were identified and then

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Fig. 1. Symptoms of leaf spots caused by *Colletotrichum boninense* on Japanese spindle tree. **A**, healthy and unhealthy leaves of Japanese spindle tree, **B**, anthracnose leaf lesion with large spots and discoloration and **C**, small and large leaf spots formed on various leaves).

deposited at Seoul National University Fungus Culture Collection (SFCC) and Centraalbureau voor Schimmelcultures (CBS). The strains were maintained at refrigerator (2-3°C) before use for cultivation. The list of taxa retrieved from GenBank for comparison study is shown in Table 1.

Morphological and cultural characteristics. Morphological observations were made from cultures grown on MEA and PDA at 25°C. For species identification, we referred to descriptions and illustrations of *C. boninense* and related species by Lu et al. (2004), Moriwaki et al. (2003) and von Arx (1981). For light microscopy, Nikon (Labophot 2, Japan) was used with differential interference contrast. Lactophenol slide mount preparation (phenol 20 g, lactic acid 20 ml, glycerol 40 ml 100 ml^{-1}) was used for staining and observing the fungi.

Stock cultures of test species were grown on PDA at 25°C and used as inocula. Growth of *C. boninense* was determined by transferring mycelial plugs (5 mm in diameter) from the growing margin of the stock cultures onto Petri dishes (90 × 15 mm in diameter). After inoculation, plates were sealed in polyethylene bags and incubated at 10, 17, 22, 25, 28 and 33°C in darkness for up to 10 days. Mycelial growth was recorded periodically by measuring diameters of the colony at right angles. The slopes of the regression lines of the linear portion of the growth curves were used for calculating growth rates.

Pathogenicity test. Pathogenicity test was conducted by inoculating slightly wounded and non-wounded leaves with a conidial suspension (1×10^6 conidia ml^{-1}) of the fungus. For each test, four detached leaves were inoculated with the conidial suspension and placed in a moist chamber at 25°C. Control leaves were sprayed with distilled water. After 3 days, symptoms on all the leaves were observed. The fungus was re-isolated from the leaves on which lesions developed after inoculation.

Table 1. List of taxa retrieved from GenBank for comparison study

Species	Strain[a]	GenBank Accessions
Colletotrichum boninense	IMI 377253	AY438552
C. boninense	IMI 376913	AY438544
C. boninense		AB087213
C. boninense		AB087214
C. boninense		AB087215
C. boninense		AB042313
C. boninense		AB051400
C. boninense		AB051402
C. boninense		AB051403
C. boninense		AB051404
C. boninense		AB051405
C. boninense	SFCC 040305	in preparation
C. caudatum		AB042305
C. cingulata	IMI 356878	AF090855
C. circinans	BBA 67846	AJ301955
C. coccodes	IMI 309371	AJ536230
C. crassipes	MAFF 712102	AB105970
C. fragariae		AB087221
C. fuscum	BBA70535	AJ301938
C. gloeosporioides (= *Glomerella acutata*)	BBA71371	AJ301982
C. gloeosporioides f. sp. *aeschynomene*	BBA71407	AJ301986
C. kahawae	IMI301220	AJ536220
C. lindemuthianum	CBS 132.57	AJ301947
C. musae		AY567968
C. orbiculare		AB042308
C. spinaciae	BBA 71333	AJ301973
C. sublineolum	MAFF 305360	AB057438
C. trichellum	BBA 71091	AJ301989
C. trifolii		AB087223
C. truncatum	PDC 005	AY266386
Penicillium decaturense	NRRL 28152	AF125946

[a] Acronyms for culture collections: BBA, Federal Biological Research Centre for Agriculture and Forestry, Germany; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; IMI, CABI now (former International Mycological Institute, Genetic Resources Collection), Egham, UK; MAFF, Ministry of Agriculture, Forestry and Fisheries Genebank, National Institute of Agrobiological Resources, Tsukuba, Japan; NRRL, ARS Culture Collection, Northern Regional Research Laboratory, U.S. Department of Agriculture, Illinois, USA; PDC, Plant Disease Clinic, Plant Pathology Department, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, USA; SFCC, Seoul National University Culture Collection, Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, Korea.

DNA extraction, amplification and sequencing. The strains were inoculated onto PDA plates and incubated at 25 °C for 4-5 days. Strains obtained from various culture collections are shown in Table 1. Total genomic DNAs were extracted from mycelia cultured on the plates covered with cellophane using AccuPrep® Genomic DNA Extraction Kit (Bioneer Corp., Daejeon, Korea). From extracted

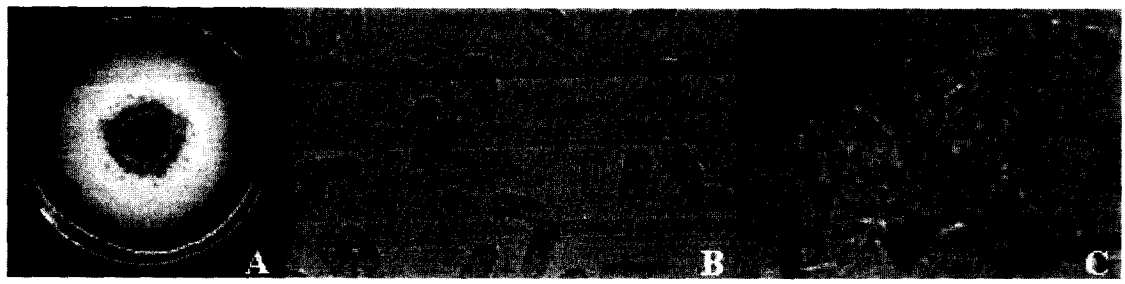

Fig. 2. Colony (**A**), conidia (**B**) and seta (**C**) of *Colletotrichum boninense* on a 9-day-old PDA culture. Each black bar represents 15 μm.

genomic DNA, the internal transcribed spacer (ITS) region 1 and 2 including 5.8S of nuclear rDNA were amplified with ITS1 and LR5 (1.5 kb) primers (White et al., 1990) using Quick PCR Premix containing *Taq* DNA polymerase, dNTPs, reaction buffer and tracking dye (GENENMED Corp., Seoul, Korea). Each PCR reaction was conducted with 30 thermal cycles according to following conditions: 1 min at 95°C for denaturation; 1 min at 52°C for primer annealing; 1 min at 72°C for extension; 10 min at 72°C for terminal extension. Amplified PCR products were detected on 0.75% agarose gel through electrophoresis. Checked amplicons were purified with AccuPrep® PCR Purification Kit (Bioneer Corp., Daejeon, Korea). The purified PCR products were sequenced with ABI3700 automated DNA sequencer (Applied Biosystems Inc., Foster, CA, USA), using ITS4 primer (White et al., 1990).

Phylogenetic analysis. For phylogenetic analysis, *Penicillium decaturense* and *C. lindemuthianum* and *C. trifolii* were used as an outgroup and a suboutgroup, respectively. Sequences generated in this study were aligned with those retrieved from GenBank using CLUSTAL X ver.1.83 (Thompson et al., 1997) with gap opening penalty 10.0 and gap extension penalty 0.02. Using PHYDIT program ver. 3.2 (Chun, 1995), ambiguous and uninformative variable sites were excluded and a sequence dataset was submitted to subsequent phylogenetic analyses. Parsimony analysis was conducted in PAUP 4.0b10 (Swofford, 2002) using tree bisection reconnection (TBR) branch swapping with MAXTREES unrestricted. All gaps were treated as missing data.

Results

Fungal description and growth. The causal fungus was purely isolated from the spot lesions of tree leaves using the blotter method. The species was morphologically characterized by its cylindrical conidia with an obtuse apex and a protruding base. The colony on PDA was whitish at margin, cream to dull orange or blackish (brown) in dots at acervuli (Fig. 2A). Conidia were formed in blackish orange masses and measured 13-17 × 5-7 μm in size (Fig. 2B).

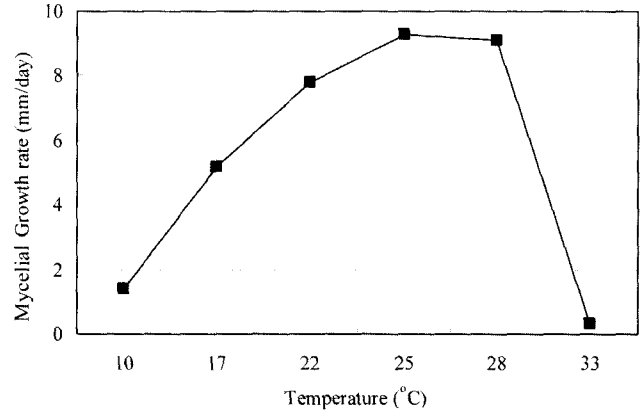

Fig. 3. Effects of temperature on mycelial growth of *Colletotrichum boninense* on PDA.

Fig. 4. A newly formed circular lesion with sporodochia on a leaf 10 days after inoculation of *Colletotrichum boninense*.

Blackish brown setae were often observed in acervuli on PDA and ranged up to 100 μm in length (Fig. 2C). Mycelial growth rate of our isolate was optimal at 25°C and measured up to 9 mm per day on PDA (Fig. 3). The morphological characteristics corresponded to the descriptions of *C. boninense* by Moriwaki et al. (2003).

Pathogenicity. Koch's postulates were fulfilled by inoculating healthy leaves of the tree with the conidial suspension. Within 7 to 10 days, symptoms similar to those

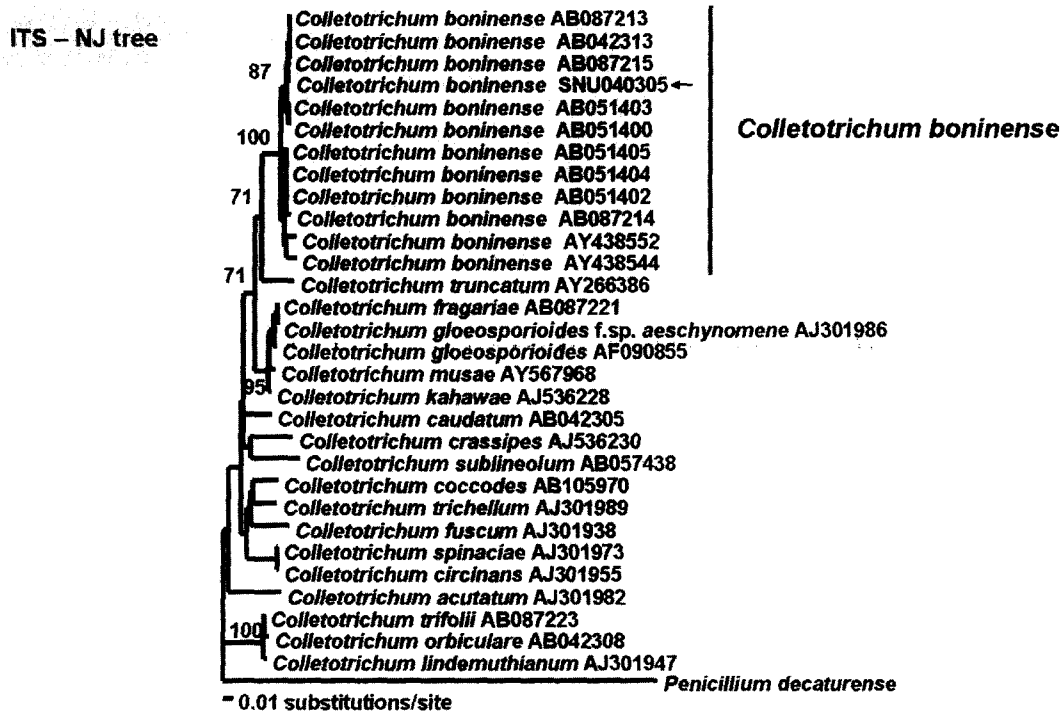

Fig. 5. NJ phylogenetic tree inferred from nuclear ribosomal ITS sequences. Bootstrap values greater than 50% are shown on corresponding branches. *Penicillium decaturense* and *Colletotrichum lindemuthianum* and *C. trifolii* were used as an outgroup and a subautgroup, respectively.

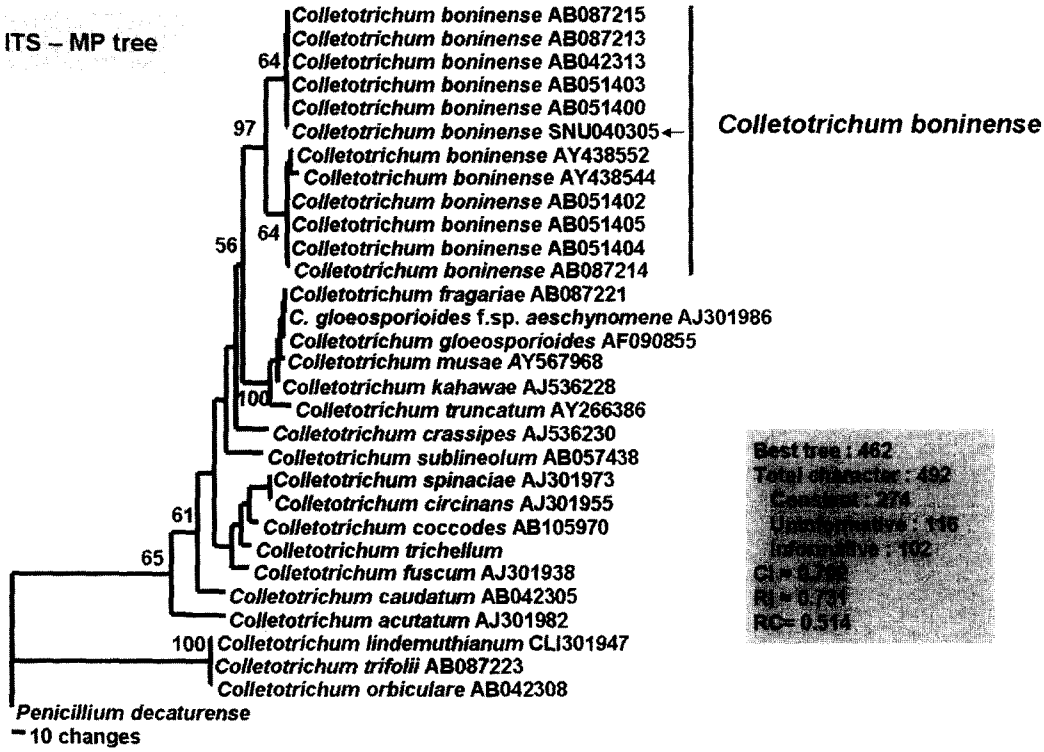

Fig. 6. MP phylogenetic tree inferred from nuclear ribosomal ITS sequences (tree length = 420, CI = 0.702, RI = 0.731, RC = 0.514). Bootstrap values greater than 50% are shown on corresponding branches. *Penicillium decaturense* and *Colletotrichum lindemuthianum* and *C. trifolii* were used as an outgroup and a subautgroup, respectively.

observed on naturally infected leaves appeared (Fig. 4). Control leaves sprayed with distilled water did not develop any symptoms. The re-isolated causal fungus was identical with that from the leaves on which lesions developed after inoculation.

Molecular phylogeny of *Colletotrichum* species. Neighbor-joining (NJ) and most parsimonious (MP) trees were constructed by using the ITS1 and ITS2 sequences, and almost the same topologies were obtained in both methods (Figs. 5, 6). As shown in Figs. 5 and 6, when the ITS sequence of the causal fungus was analyzed with 11 isolates of *C. boninense* retrieved from GenBank, the intraspecific DNA homologies were 97.4 to 99.6%. Trees made by the NJ and MP methods also indicated the monophyly of *C. boninense* strains. When compared with related species such as *C. gloeosporioides*, *C. musae*, *C. fragariae*, *C. spinaciae* and *C. lindemuthianum*, the interspecific DNA homologies were 93.9, 91.7, 93.6, 92.6 and 88.7%, respectively. MP analysis generated 462 MP trees with tree length of 420 steps, with a consistency index (CI) = 0.702, a retention index (RI) = 0.731 and a rescaled CI (RC) = 0.514. Molecular phylogenetic analysis on ITS sequences clearly distinguished the species from *C. gloeosporioides* as well as other similar *Colletotrichum* species such as *C. musae* and *C. fragariae*. On the basis of morphological and molecular characteristics, the causal fungus was identified as *C. boninense* Moriw., Sato & Tsukib.

Discussion

Recently, *C. boninense* was found to inhabit a wide range of host plants such as *Crinum*, *Clivia* and *Cymbidium* and distribute on the Pacific Coast of Japan (Moriwaki et al., 2003). Crous et al. (2004) also mentioned the occurrence of the species on Proteaceae. The species previously fell within the broad species concept of *C. gloeosporioides*, but is now differentiated by morphological characteristics and molecular phylogenetic analyses of ITS sequences (Lu et al., 2004; Moriwaki et al., 2003). Moriwaki et al. (2003) presented that the interspecific DNA homologies with related taxa were 80.2 to 82.3% for *C. gloeosporioides*. When ITS sequences of our fungus were phylogenetically analyzed with related *Colletotrichum* species, the interspecific DNA homologies were low and ranged 88.7% to 93.9. Molecular phylogenetic analyses of ITS sequences clearly distinguished *C. boninense* from *C. gloeosporioides* as well as other similar *Colletotrichum* species such as *C. musae*, *C. fragariae*, *C. spinaciae* and *C. lindemuthianum*.

Thus, morphological distinction and monophyly based on the molecular phylogenetic analyses of ITS regions verified the taxonomic indentity of our fungus as *C. boninense*. In Korea, it was once reported that *Gloeosporium euonymicola* Hemmi causes anthracnose on Japanese spindle tree (Korea Forest Research Institute, 1991). In this study, another fungus, *C. boninense*, was found to cause anthracnose on Japanese spindle tree in Korea. *Colletotrichum* (imperfect state of *Glomerella*) differs from *Gloeosporium* (conidial state of *Glomerella*) in having setae, which may be absent in some cultures (Barnett and Hunter, 1987). This is the first study on the pathogenicity, mycelial growth and molecular phylogenetic characteristics of *C. boninense* isolated from Japanese spindle tree. However, as shown in Figs. 5 and 6, there are many other strains in *C. boninense*, suggesting that there can be various unknown hosts. Thus, further studies on the host range, geographical distribution, and ecological characteristics of the fungus are needed.

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